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Keywords

Antibiotic resistance, antimicrobial peptides, efflux pumps, Gram-negative, lipopolysaccharide, nosocomial, nanomaterial, outer membrane, polymyxin, porins.

Abstract

Antimicrobial resistance is one of the leading threats to society. The increasing burden of multidrug-resistant Gram-negative infection is particularly concerning as such bacteria are demonstrating resistance to nearly all currently licensed therapies. Various strategies have been hypothesized to treat multidrug-resistant Gram-negative infections including: targeting the Gram-negative outer membrane; neutralization of lipopolysaccharide; inhibition of bacterial efflux pumps and prevention of protein folding. Silver and silver nanoparticles, fusogenic liposomes and nanotubes are potential strategies for extending the activity of licensed, Gram-positive selective, antibiotics to Gram-negatives. This may serve as a strategy to fill the current void in pharmaceutical development in the short-term. This review outlines the most promising strategies that could be implemented to solve the threat of multidrug-resistant Gram-negative infections.

Introduction

There is a drastic need for innovative therapeutic solutions that selectively target multi-drug resistant Gram-negative infections. Resistance can be attributed to nearly all conventional antibiotics used clinically, and there are a lack of effective antibiotics in reserve. Gram-negative bacteria, particularly: *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, are an ever-increasing threat to health and

particularly that of hospitalized patients who are commonly immunocompromized, have co-morbidities and are less able to fight infection [1]. Recently, emphasis has been placed on the successful and rapid detection of specific, causative antimicrobial resistant strains and the significance this will have on reducing antimicrobial associated mortality. Research has increasingly focused on the development of pathogen-specific, narrow-spectrum antimicrobials and novel preventative methods for antimicrobial infections, for example vaccines. Vaccine development is largely responsible for the successful management of a range of infectious diseases in the developed world including: polio, measles, diphtheria and tetanus. A vaccine-based approach may also reduce the requirement for antibiotic therapy in bacterial infections that occur secondary to viral infection [2]. This change in focus, from broad-spectrum microbial annihilation to more targeted therapy, acknowledges the major contribution empirical prescribing has on increasing drug resistance and its impact on beneficial human microbiota [3].

Nosocomial infections are a major contributor to healthcare associated infections and antimicrobial resistance. Approximately 20-40% are attributed to the transfer of commensal microorganisms from the skin of healthcare workers to patients or derived from the patients' own commensal flora [4]. Healthcare associated infections affect approximately 4.1 million patients annually within the European Union. They are a major contributor to morbidity and mortality, causing 37, 000 deaths annually and a further 100,000 deaths in those with co-morbidities [5]. In terms of antimicrobial resistant infections, a recent UK government report estimate that these contribute to around 25,000 deaths annually in Europe alone [6].

Gram-negative bacteria are a particular problem due to multiple inherent resistance mechanisms, most notably the presence of a lipopolysaccharide (LPS) outer membrane and efflux pumps [7]. As a result of improper and overuse of antimicrobials, the resistance rates to current therapeutic agents have increased to serious levels. This dilemma has attracted the attention of scientists, the general public, health authorities and politicians. It is now recognized as a considerable global health problem [4]. As mentioned, the significant lack of newly licensed antimicrobial pharmaceuticals translating from the laboratory to patients is concerning. In the past 25 years, only two new cephalosporin-beta(β)-lactamase inhibitor combinations: ceftolozane/tazobactam in 2014 and ceftazidime/avibactam in 2015, have been approved to treat systemic bacterial infections caused by multi-drug resistant Gram-negative bacteria [8]. There are a multitude of reasons for the decline in antimicrobial drug development, most notably the high financial commitment and time required for developing and registering a new drug. On average it costs approximately $800 million to introduce a new drug to market with development times normally in excess of 10 years. Parallel to this, the pharmaceutical industry has focused over the past 30 years on therapies for chronic diseases such as diabetes and cardiovascular disorders. These products are likely to be required as lifetime treatments in contrast to antibiotics that are most commonly short-term acute treatments (typically 5-14 days) providing a more reliable market for generating profits [9]. An optimal scenario would be one were newly approved antibiotics are held in reserve for infections that are resistant to commonly employed therapies in line with a fully defined antibiotic formulary. Companies who develop novel antibiotics may ensure returns on their investments via schemes such as patent extensions. However, such approaches encounter difficulties as generic manufacturers in particular express reservations about extending antibiotic patents. Cheaper, off-patent, generic antimicrobials are under-regulated, especially in developing nations, increasing both the development of resistance and the expectation

from healthcare providers that all, including new antibiotics, should be priced similarly [10]. Other restrictive factors include clinical trial requirements, particularly the challenge of proving novel therapies produce greater clinical outcomes compared to existing products, and that they are sufficiently safe for use. In order to increase the approval and registration of new antimicrobials, the US Food and Drug Administration have indicated that it may be ready to alter its strict clinical trial requirements and reassess the antimicrobial approval regulations in order to increase the potential availability of novel treatments [11]. The primary barriers to overcome, as will be discussed further in this review, include the specific targeting of Gram-negative bacteria in order to produce selective antibiotics that are suitable candidates for clinical trials and transition of these molecules from the laboratory to the clinic.

The Gram-negative outer membrane as a barrier to therapy

I. Bacterial cell wall structure

Understanding the mechanisms that govern Gram-negative bacterial resistance requires a fundamental appreciation of their cell morphology. The unique structure of the outer membrane of Gram-negative bacteria plays an important role, providing an additional layer of mechanical protection, without affecting the selectivity or exchange of material needed for bacterial survival [12]. The Gram-negative cell wall is composed of an outer LPS membrane and an inner cytoplasmic membrane. A thin layer of peptidoglycan and lipoproteins exist within the periplasmic space. The inner cell membrane is composed of a phospholipid bilayer, whilst the outer membrane consists of phospholipids within its interior leaflet and a LPS outer leaflet [13]. Porins and specialized transporters are also present within the outer membrane channels and mediate the influx of a variety of compounds including nutrients and minerals such as sugars, amino acids, phosphates and ions. Porins play an important role in

bacterial metabolism and growth, and are therefore a valuable target for antimicrobial drug development [14]. Gram-negative bacteria continuously alter the expression and function of outer membrane porins, hence this may affect their sensitivity to antimicrobial agents. Loss of or changes in porin amino acids influence the ability or rate of entry of antibiotics and contribute to such resistance. In contrast to Gram-negative bacteria (Figure 1a), Gram-positive bacteria lack an outer membrane and are composed of a single lipid membrane surrounded by numerous interconnecting layers of peptidoglycan and lipoteichoic acid (Figure 1b) [15]. Although Gram-positive bacteria possess a cell membrane, the lack of a protective outer membrane makes them more susceptible to antibiotics. The outer surface of both Gram-positive and Gram-negative bacteria is protected by an external capsular layer, which acts as an additional barrier to antibiotic therapy. The capsule is composed of long chains of negatively charged polysaccharides, defined as capsular polysaccharides. In some *E. coli* isolates, capsular polysaccharides can extend from the cell surface for approximately 100-400 nm. Capsular polysaccharides can also aid bacterial evasion from the host immune response by limiting the activity of phagocytes and increasing bacterial pathogenicity [16].

II. Antimicrobial resistance mechanisms of Gram-negative bacterial cell wall

The outer membrane of Gram-negative bacteria acts as a selective barrier by adding a hydrophobic lipid bilayer to the specific size-exclusion properties of porins. The outer membrane has the ability to block the entry of numerous toxic compounds and prevent the uptake of molecules with a molecular mass greater than 600 Daltons [17]. The influx of metabolites such as sugars, phosphates and hydrophilic molecules is mainly directed by porins. The continuous alteration in lipid or protein composition of the outer membrane leads

to drug-resistance. This involves increasing outer membrane hydrophobicity, changing porin specificity or increasing the number and efficacy of efflux pumps [18].

Reducing the negative charge of LPS within the bacterial outer membrane is one of the key strategies employed by Gram-negative bacteria to negate the action of membrane active cationic antimicrobials, such as chlorhexidine and cationic antimicrobial peptides. This is achieved via the addition of positively charged residues such as aminoarabinose and galactosamine sugars to LPS or by the removal of negative charged moieties. This modification leads to increased bacterial survival as demonstrated by both *P. aeruginosa* and *Francisella novicida* after exposure to the cyclic cationic lipopeptide polymyxin B [19]. Amines are also harnessed by Gram-negatives to increase LPS membrane cationicity as demonstrated by *Salmonella typhimurium*, which increases tolerance to polymyxin B by conjugating phosphoethanolamine to one of the phosphate groups present within outer membrane lipid A [20]. Bacteria are also able to remove anionic phosphate groups to reduce the overall anionic surface charge of LPS, proven by the removal of the 4′-phosphate group from lipid A in *Helicobacter pylori*. This results in increased resistance to membrane active cationic antimicrobial peptides [21]. Phospholipids present in the Gram-negative outer membrane are also susceptible to modification. *S. typhimurium* has the ability to increase the levels of outer membrane glycerophospholipids resulting in increased membrane hydrophobicity and reducing the permeability of charged, water soluble molecules [22].

Alteration of outer membrane porins prevent intracellular diffusion of small hydrophilic antibiotics such as β-lactams, tetracycline, chloramphenicol and fluoroquinolones. Research has revealed that functional changes in porins are directed by specific mutations in a variety

of pathogens, including *E. coli*, *P. aeruginosa* and *Neisseria gonorrhoeae* [14, 23]. A relatively minor change in porin structure can have a significant effect on functionality. For example in *Enterobacter aerogenes*, substitution of glycine with aspartate within the peptide structure of its porin results in a narrower lumen, affecting intracellular cephalosporin transport and lowering susceptibility to antimicrobials [14]. A reduction in porin expression is responsible for carbapenem resistance in extended-spectrum β-lactamase producing *K. pneumoniae* and *E. coli* isolates. Susceptibility to carbapenems, such as ertapenem, was reduced further when porin loss was combined with an increased production of extended-spectrum β-lactamases [24].

Efflux pumps are membrane bound proteins that regulate intracellular active transport mechanisms to extrude toxic compounds, such as bile salts, fatty acids and heavy metals, out of bacterial cells [25]. They are important cellular machinery and increase Gram-negative bacteria's ability to resist diverse classes of antibiotics including β-lactams, aminoglycosides and fluoroquinolones via expulsion. These antibiotics often possess intracellular targets hence their expulsion restricts their activity. Efflux pumps also contribute to bacterial virulence and the formation of biofilms [26]. The resistance-nodulation-division family (RND), one of five families of bacterial efflux pumps, is the only family that is specifically associated with Gram-negative bacteria. Other families of efflux systems are extensively found in both Gram-positive and Gram-negatives [27]. RND efflux pumps are able to expel a wide range of antibiotics with a high degree of specificity. MexAB-OprM and MexXY-OprM are RND-based efflux pumps common to *P. aeruginosa*. MexAB-OprM and MexXY-OprM can expel tetracycline, fluoroquinolones, and chloramphenicol, whilst for β-lactams and novobiocin, expulsion occurs only via the MexAB-OprM system [26]. Gram-negative bacteria also have the ability to increase the expression of anionic capsular polysaccharides leading to increased

binding of cationic antibacterials, for example polymyxins, via electrostatic interactions. This results in a reduction in the concentration and therefore in the activity of polymyxins at bacterial cell membranes [19].

Strategies for extending therapeutic activity against Gram-negatives

I. Antimicrobial peptides

Antimicrobial peptides were first isolated by Dubos in 1939 from *Bacillus* bacteria derived from soil [28]. The amphipathic nature of most antimicrobial peptides proves advantageous for antimicrobial activity. The presence of hydrophilic and hydrophobic domains allow interaction with both lipid and phospholipid groups present in the bacterial cytoplasmic membrane [29]. The majority of antimicrobial peptides are cationic in character. These naturally occurring molecules mediate innate immunity in a multitude of organisms [30]. They possess several optimal properties for therapeutic applications. Cationic antimicrobial peptides have the ability to bind to LPS and therefore negate the production of host pro-inflammatory cytokines [31]. Most cationic antimicrobial peptides exert a cidal action via targeting of bacterial membranes, resulting in membrane disintegration, cell lysis and death [30]. A variety of antimicrobial peptides also demonstrate an ability to permeate bacterial cell membranes at low concentrations, inhibiting DNA replication and protein synthesis without altering membrane integrity [29]. For example, buforin-II binds to DNA and RNA without disrupting the bacterial cell membrane architecture [32]. Cationic antimicrobial peptides have great potential to fill the current void in antimicrobial drug development because of their selectivity for negatively charged microbial membranes compared to neutral, sterol-rich mammalian forms. Antimicrobial peptides tend to demonstrate rapid bactericidal activity utilizing multiple modes of extra- and intra-cellular action. They therefore have a reduced

tendency to promote bacterial resistance compared to many currently licensed antimicrobials which tend to target only a single biomolecular mechanism. Antimicrobial peptides are already in clinical use, including lysostaphin, polymyxin B and gramicidin S, demonstrating their potential for clinical translation and ability to fill the current void in antimicrobial drug development [33].

Polymyxins are a class of cationic cyclic lipopeptides, first discovered in 1947, isolated from the spore-forming bacteria *Paenibacillus polymyxa* present in soil. Polymyxin E (colistin) and polymyxin B are classified as narrow-spectrum Gram-negative selective antibiotics. Their clinical use decreased in the 1970s due to concerns regarding nephro- and neuro-toxicity. Most recently there has been a revival in their potential clinical use and research has focused on the design of novel polymyxin derivatives with markedly lower mammalian toxicity and higher bactericidal activity [34]. The exact bactericidal mechanism of polymyxins has remained a topic for debate amongst researchers. It has been hypothesized that the protonated amino acids within the cyclic peptide structure of polymyxins, bind directly to the lipid A part of LPS present in the outer membrane of Gram-negative bacteria, facilitating insertion of hydrophobic motifs into the outer membrane. This enables the formation of pore-like aggregates, thus increasing outer membrane permeability [35]. Polymyxin B, for example, has the ability to attach to the anionic surface of LPS in the outer membrane resulting in self-promoted uptake into the periplasmic space and cytoplasmic membrane. It is more difficult for bacteria to generate resistance against such physical interactions as it would require reorganisation of vast areas of the membrane architecture. However, plasmid-borne resistance has been reported recently against colistin and this is concerning as colistin is typically considered a drug of last resort for Gram-negative infections [36]. The *mcr*-1 plasmid, identified in an *E. coli* isolate present in a pig in China, encodes an enzyme that directs the

addition of phosphoethanolamine to lipid A, decreasing the anionic charge of the outer membrane. Whilst this addition has been elucidated previously, the fact that the process is mediated via a plasmid is crucially significant, as it will allow resistance to readily spread to other species. This discovery highlights the urgent need for investment to fully elucidate antimicrobial resistance mechanisms and thereby create tailored therapies to combat these.

Research into polymyxin-like molecules has been on-going, especially with regard to producing less toxic derivatives (nephro- and neuro-toxic) and compromising the integrity of the Gram-negative outer membrane barrier to increase the activity of existing antibiotics [37]. Structurally similar cyclic antimicrobial peptides are also of interest as future synthetic therapies as they possess increased serum stability relative to linear forms. They may also provide a basis for designing cost-effective, low molecular mass, anti-LPS compounds [38]. Cyclic peptide variants are synthesized by directly conjugating the two terminals of a primary amino acid sequence to form an amide bond, or via another form of linkage such as lactone or disulfide bonds. Generally, cyclic peptides are more effective than their linear analogues because of their structural rigidity, whichenables them to bind selectively to bacterial targets. They can also adopt an ordered amphipathic structure that allows them to insert deeper within the bacterial membrane abd possess an extended duration of action *in vivo* due to their increased stability to proteases [39]. Almost all known natural cyclic peptides display potent antibacterial activity. For example, polymyxin B, colistimethate and gramicidin S show high bactericidal activity against *P. aeruginosa* with minimum bactericidal concentrations of 0.125, 4 and 8 μg/ml respectively [40]. Despite their significant bacterial activity *in vitro*, many cyclic peptides are highly haemolytic and currently lack the bacterial selectivity required for clinical translation [41].

There are several limitations associated with the clinical application of antimicrobial peptides. These include: *in vivo* toxicity; difficulties encountered upscaling synthesis to pharmaceutical manufacturing requirements; their short *in vivo* half-life and reduced bioavailability due to proteolytic degradation [33]. Various strategies have been employed to overcome these obstacles. Researchers have performed several chemical modifications to improve the biological stability of peptides. Such approaches include: substituting naturally occurring L-amino acids with their respective D-enantiomers; switching natural amino acids with unnatural variants, for example switching lysine with ornithine; and the synthesis of peptidomimetics, including peptoids. In addition to chemical modification, the use of polymeric carriers and delivery systems have also been shown to enhance the bioavailability of peptide formulations. These include: the use of mucoadhesive polymers; conjugation of peptides to poly(ethylene glycol) (PEGylation), glycosylation, and the development of peptide-based hydrogel, nanoparticle, emulsion and liposomal formulations [42].

II. Combinational antibiotic treatment for Gram-negative bacteria

Synergistic therapy, a combination of two or more antibiotics, is a commonly employed strategy to resolve Gram-negative infections. In comparison to monotherapy, combination therapy takes advantage of the additive effects of multiple antimicrobial mechanisms for each drug therapy to lower the risk of resistance developing. Combination therapy may also lower mortality and improve clinical outcomes. It is recommended for patients whose infection is suspected or confirmed to be caused by multidrug-resistant Gram-negative bacteria [43]. Synergy between two or more antimicrobial agents means that their combined effects will be greater than their individual effects. Generally each individual antibiotic employed varies

285 with respect to their mode of action [44]. However, the use of multiple therapies does not
286 come without risk. Combination therapy has been associated with an increase in
287 nephrotoxicity, especially when prescribed for long-term chronic infections. Another
288 disadvantage is the increased complications associated with multiple treatment schedules
289 [45]. Combinational therapy is a successful strategy for resolving *Mycobacterium*
290 *tuberculosis* infection and has demonstrated a significant impact in decreasing the
291 development of rifampicin resistance. However, the majority of clinical studies show no
292 difference in the emergence of resistance during treatment of Gram-negative infections with
293 dual therapy versus monotherapy [45]. High risk and critically ill patients who present with
294 severe infections such as; pneumonia, neutropenia and septic shock commonly require rapid
295 initiation of broad-spectrum empirical therapy. Inclusion of more than one antibiotic can
296 improve clinical outcomes by ensuring at least one agent is active against the unknown
297 pathogen(s). Once the causative isolate has been identified and the susceptibility tests
298 completed, narrow-spectrum therapy with single active agent is optimal [45]. A model
299 combination therapy includes a broad-spectrum β-lactam with an aminoglycoside, macrolide
300 or fluoroquinolone for treatment of *Pseudomonas* infections [43]. A novel combination
301 between a cephalosporin and a β-lactamase inhibitor has been recently approved [8]. An
302 appreciation of the pharmacokinetics/pharmacodynamics (PK/PD) profile against the
303 causative microorganism(s) is necessary in order for infection to be successful resolved. The
304 development of innovative PK/PD models, *in vitro* studies, animal and computer models and
305 previous patient data can improve the rational use and predicted clinical outcomes of
306 combined therapies. The PK/PD parameters that determine the cidal activity of antibiotics are
307 either concentration-dependent or concentration-independent, deemed time-dependent.
308 Optimizing antibiotic dosage regimens using *in vitro* models and prioritizing PK/PD
309 parameters will lead to improved clinical outcomes; reduced toxicity and limit the potential

for sub-therapeutic concentrations of antibiotics reaching the infection site, a major risk for the development of resistant pathogens [46].

III. The activity of silver against Gram-negative bacterial infection

Silver has been known to protect against infection for over 2,000 years and continues to be used widely in many antimicrobial applications, especially within the biomaterial industry. Morones-Ramez and colleagues demonstrated that silver ions (Ag^+) have a synergistic effect with β-lactam, aminoglycoside and quinolone antibiotics against a variety of Gram-negative bacteria. Silver has been shown to increase the production of reactive oxygen species, including hydroxyl radicals (OH•), increasing the permeability of the outer membrane to commonly employed antibiotics [47]. Silver also acts intracellularly to inactivate bacterial protein synthesis and enzymes responsible for a range of biochemical processes, including deoxyribonuclease and ribonuclease. Silver has also been implicated in DNA degradation and activation of cysteine proteases, namely the cysteine-dependent aspartate-directed proteases, which play an important role in bacterial cell apoptosis. Silver ion's cationic properties bestow affinity for anionic minerals present in the host, such as chloride or phosphate, or proteins such as albumin. The complexes that form are inactivated by precipitation or deposit in tissue debris with the potential to cause toxicity. Problems such as these have led to questions regarding the safety and widespread use of silver for antibacterial applications. More recently studies have focused on improving silver's ability to selectively target bacterial metabolic pathways via silver nanoparticle systems [48]. Silver nanoparticles have attracted interest in the development of new pharmaceutical products. They have been introduced into wound dressings, medical device coatings, and are increasingly utilized as drug delivery nanomaterials. Silver nanoparticle dressings, when compared to silver sulfadiazine cream, have been found to decrease wound-healing time and improve the clearance of bacteria from the infection site. Within medical devices, silver nanoparticles have been tested as novel

coatings for catheters, which are typically liable to bacterial infections leading to complications such as device failure and sepsis. Furthermore, they have the potential to be administered as drug delivery platforms, acting as carriers for licensed antibiotics and enabling penetration of the Gram-negative outer membrane [49].

Specific methods to target Gram-negative pathogens

As highlighted, the development of bacterial resistance towards existing antimicrobial agents has led to an urgent need for effective, alternative strategies. There is a necessity to develop novel classes of antibiotics and different methods to bypass current mechanisms of Gram-negative resistance [7]. There are multiple hypothesized mechanisms by which this can be achieved including: targeting membrane integrity by binding to LPS; interacting with the DsbA-DsbB enzyme system; or blocking the intracellular expulsion of antibiotics via inhibition of efflux pumps. Innovative drug delivery platforms are also considered to be "smart" approaches to enhance the efficacy of existing and future antibiotics. Genetic engineering of phage lytic enzymes is also a promising strategy with the potential to kill specific Gram-negative bacterial strains. Whilst all these approaches hold great promise, their potential for pharmaceutical scale-up and related regulatory barriers have to be considered early in the drug development process. Additionally, the high cost and the requirement to prove quality, efficacy and safety considerations are the main reason behind clinical trial failure and cessation of antimicrobial drug development [50]. Despite these regulatory hurdles, this review will look further at the most promising approaches to resolving the clinical and resistance barriers that govern Gram-negative bacterial infection.

I. Negating the biological effects of Gram-negative lipopolysaccharide

Besides being the major constituent of the outer membrane, LPS signals bacterial invasion and triggers an aggressive host immune response resulting in the release of pro-inflammatory mediators, cytokines, chemokines, and lipoproteins [51]. Lipid A is the hydrophobic portion of LPS that is chiefly responsible for biological toxicity. Within the outer membrane it protects Gram-negative bacteria from host immune defenses by forming a gel-like layer of low fluidity. This layer limits the influx of hydrophobic solutes into the cell, including many antibiotics [52]. Excessive host response to LPS causes organ dysfunction, septic shock and can even result in death. Antibiotics currently used to treat Gram-negative infections exacerbate the immune crisis by causing bacterial cell lysis, resulting in the release of significant amounts of LPS and intracellular toxins into the systemic circulation and creating an infection that is difficult to treat effectively [53]. The risk of such events requires consideration prior to initiation of empirical therapy as demonstrated in 2011, when the European Union witnessed a haemolytic uremic syndrome outbreak caused by Shiga toxin-producing *E. coli* O104:H4. Treatment with antibiotics, such as quinolones, enhanced the release of intracellular Shiga toxin resulting in multiple deaths [54].

The severity of the host response is mediated by plasma lipoproteins and the LPS-binding receptor CD14 that appears on the surface of host macrophages and neutrophils [55]. Examples of plasma lipoproteins include lipopolysaccharide-binding protein (LBP), bactericidal/permeability-increasing protein (BPI), phospholipid transfer protein and antimicrobial proteins secreted by neutrophils. Their binding to LPS causes a variety of cellular effects [56]. Both soluble LBP and CD14 are present in the blood and are known to enhance the effects of bacterial LPS. When LPS binds to LBP, the complex is recognized by host CD14 receptors that in turn activate the production of pro-inflammatory cytokines and type-I interferon, leading to local and systemic inflammatory reactions [55]. By contrast, BPI

binding to LPS is thought to be inhibitory and therefore beneficial in preventing an exaggerated immune response. Recombinant and modified forms of BPI have been assessed in clinical trials in patients with severe sepsis or septic shock. For example, recombinant BPI ($rBPI_{21}$) is composed of the amino-terminal half of naturally occurring BPI and possesses antibacterial and anti-LPS effects. When one amino acid cysteine residue of BPI is replaced with alanine biological stability is significantly improved without affecting the neutralizing properties of BPI [57]. This highlights how naturally occurring biomolecules can be altered synthetically to improve pharmacological and pharmaceutical properties. If harnessed correctly it will enable a wealth of potential therapies to be explored.

Throughout history nature has been the most significant source of antimicrobial therapies and there has been an increased focus on identifying novel molecules of interest from natural sources. Limulus anti-LPS factor (LALF) is an example of a small cyclic basic peptide found in haemocytes of marine chelicerates, demonstrating a strong affinity to LPS. It shows the ability to neutralize LPS by inhibiting the inflammatory cytokine tumour necrosis factor-alpha produced as a result of LPS stimulation of the immune response. The amino acid sequence that is responsible for LALF activity is found between amino acids 31 and 52 within the primary peptide sequence. The synthetic peptides derived from LALF 31-52 bind to LPS with high affinity and inhibit binding of LPS to LBP in a dose-dependent manner. The protective effect of LALF has been shown *in vivo* via *E. coli* and *P. aeruginosa* sepsis models in mice, with administration of LALF resulting in extended life span and decreased mortality [58].

II. Targeting disulfide bond formation by the bacterial DsbA-DsbB enzyme system of Gram-negative bacteria

The folding, stability and activity of a multitude of proteins in prokaryotic and eukaryotic cells are attributed to disulfide bonds formed between pairs of cysteines within peptide monomer units. Formation of a covalent disulfide bridge, via oxidation of sulfhydryl groups (-SH) on corresponding cysteines, is important for the stabilization of the protein tertiary structure. In bacteria disulfide bond formation is mediated by the DsbA-DsbB enzyme system. The Gram-negative bacterial genotype encodes for a diversity of cysteine-based disulfide bound proteins that are responsible for many bacterial virulence factors including toxins, adhesins, flagella, fimbriae, and secretion systems [59]. For example, *E. coli* has around 300 proteins consisting of even numbers of cysteine residues that require DsbA for folding [60]. It is hypothesized therefore that inactivation of enzymes that mediate the creation of disulfide bonds in such proteins will disturb the stability and activity of related virulence factors.

In Gram-negative bacteria the periplasmic enzyme DsbA is a member of the thioredoxin family and oxidizes complementary pairs of cysteines to form disulfide bonds during their movement through the cytoplasmic membrane into the cell envelope (Figure 2) [59, 61]. The resulting reduced active site cysteine of DsbA is re-oxidized by the inner membrane partner protein DsbB, restoring DsbA's activity. The subsequent reduced DsbB is reoxidized and restored using the oxidizing power of membrane-embedded quinones [62]. A number of molecules have been found that disrupt this enzymatic pathway. Landeta and colleagues discovered during high throughput screening that 4,5-dichloro-2-(2-chlorobenzyl)-3(2H)-pyridazinone inhibits disulfide bond formation in *E. coli* by blockage of the DsbB enzyme *in*

vitro. This compound was shown to bind covalently to the DsbB-DsbA system and inhibit *E. coli* growth. 4,5-dichloro-2-(2-chlorobenzyl)-3(2H)-pyridazinone was also shown to inhibit DsbB enzymes in eight of the nine Gram-negative pathogenic bacteria studied [59]. Since the DsbA-DsbB system is responsible for disulfide bond formation in Gram-negatives, it is an essential process for the correct folding and assembly of multiple virulence factors and the bacterial cell envelope. This makes it a key target for the development of new drugs to tackle Gram-negative infection. These compounds also exhibit synergistic effects with a variety of antibiotics including β-lactams, kanamycin, erythromycin, novobiocin, and ofloxacin [63].

III. Inactivating Gram-negative efflux pumps

RND efflux pumps in Gram-negative pathogens play an important role in bacterial resistance to a wide range of antibiotics, and so they are considered as a valuable field for development of efflux pump inhibitors (EPI) for use in combination therapy. EPIs are envisaged to increase the intracellular retention time and therefore efficacy of co-administered antibiotics [64]. As outlined previously, RND pumps in Gram-negative bacteria are responsible for exporting drugs and other toxic cations out of the cell. Their expression is upregulated in response to external stress factors, including reactive oxygen species, cell membrane injury or ribosome blocking agents [65]. The main RND efflux pumps expressed in Gram-negatives are AcrAB-TolC in *E. coli* and MexAB-OprM in *P. aeruginosa*. Located within the inner cell membrane, their efflux action is mediated by bacterial periplasmic adaptor proteins and an outer membrane channel (Figure 3). If an antimicrobial agent successfully transverses the outer membrane, via diffusion or porin channels, it enters the periplasmic space. Once the antibiotic is in the periplasmic space it binds to the substrate-binding pocket of periplasmic adaptor proteins. The drug is actively transported to the outer membrane channel and into the

extracellular environment. *P. aeruginosa* PAO1 alone has 12 different RND efflux systems demonstrating the varying complexity of bacterial efflux systems and the significant contribution they have to Gram-negative resistance [64].

The physicochemical properties of the antibiotic molecule also determines its extrusion rate by efflux pumps. RND pumps are primarily composed of an amino acid sequence with lipophilic side chains. Small hydrophilic molecules, which move rapidly through porins, possess a low efflux rate limiting their expulsion from the periplasmic space. However in *P. aeruginosa*, porins only allow a much slower entry of small molecules and so efflux pumps can rapidly export them out of the cell. RND pumps also effectively efflux more lipophilic and larger molecules, as they diffuse slowly through the hydrophobic layer of the outer membrane. The rate of influx and active efflux of a drug can influence the minimum inhibitory concentration (MIC) of an antibiotic *in vitro* [66].

Researchers have attempted to inhibit RND efflux pumps to restore the activity of antibiotics previously deemed unusable due to the development of resistance [65]. Peptidomimetic molecules were the first synthesized EPIs. Phenylalanyl-arginyl-β-naphthylamide is a peptidomimetic compound that inhibits levofloxacin efflux in *P. aeruginosa* overexpressed with MexAB-OprM efflux pumps. It achieves this by directly competing with the antibiotic sites on MexAB-OprM [22]. Another novel EPI is the pyranopyridine derivative, MBX2319, which increases *E. coli* sensitivity to ciprofloxacin, levofloxacin and piperacillin by inhibiting AcrAB-TolC efflux pumps. Peptidomimetic EPIs often possess cidal antibacterial activity alone but are more likely to form an important role within future clinical strategies as part of combination therapy. Unfortunately the clinical translation of such peptidomimetics has been

restricted due to toxicity concerns. For example phenylalanyl-arginyl-β-naphthylamide demonstrates prolonged accumulation in tissues and is shown to be it was toxic to renal cells. No efflux pump inhibitor has yet received marketing authorization for widespread therapeutic use, as researchers aim to improve their clinical potency and safety [66].

Methods to extend the spectrum of activity of existing narrow-spectrum Gram-positive antibiotics to Gram-negatives

The majority of antimicrobial agents, especially within the field of antimicrobial peptides, characterized in the laboratory setting are more active against Gram-positive than Gram-negative bacteria [30]. A similar scenario exists clinically with a worrying lack of effective treatment options in reserve. Of greatest significance is the increase in resistance attributed to the Gram-negative pathogens *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii*, due in part to a lack of available, effective narrow-spectrum Gram-negative selective antibiotics. The majority of antibiotics reserved for resistant Gram-positive infection have no activity against Gram-negatives as they are incapable of crossing the Gram-negative LPS outer membrane barrier. The critical need for urgent action in the licensing and availability of effective antimicrobials to treat Gram-negative infections clinically has led researchers to concentrate their efforts on uncovering new and effective drug delivery systems to expand the spectrum of activity of currently licensed antibiotics. One of the most promising areas in drug development is the design of antisense antibacterial molecules. These are synthetic DNA mimics, composed of 10 to 20 nucleic acid bases, which target messenger RNA (mRNA) and inhibit bacterial gene expression. Synthetic antisense molecules also include peptide nucleic acids, phosphorothioates and phosphorodiamidate morpholino oligomers. They have the ability to selectively target genes essential for bacterial survival resulting in inhibition of

bacterial growth, death or restoration of bacterial susceptibility to antibiotics (Figure 4). However, as the majority of antisense oligomers are hydrophilic macromolecules, they have difficulty crossing the outer membrane of Gram-negative bacteria. The coupling of short amphipathic peptides to antisense molecules has been proposed to resolve this issuewith further studies warranted [68].

Highlighting the importance of combating antimicrobial resistance, a variety of innovative platforms are currently in development. Comprehensive reviews by Long and Williams [69] and Czaplewski and colleagues [70] outline in further detail novel antimicrobials in preclinical and clinical development including: wild-type and engineered bacteriophages and siderophore-mimetics with the potential to overcome Gram-negative outer membrane barrier. The development of a novel siderophore cephalosporin S-649266, which possesses a catechol moiety on its side chain has been particularly promising and is currently undergoing phase II clinical trials [71]. The catechol siderophore was demonstrated to increase penetration of the outer membrane by carrying ferric ions into the cell via an iron transporter pathway. This review will focus mainly on the development of engineered phage lysins, fusogenic liposomes and nanotubes. All of these platforms represent promising approaches to tackle the current deficit in Gram-negative antibiotics. The immediate hope is to extend the currently available antibiotic library using regulatory approved Gram-positive drugs.

I. Fusogenic liposomes

Liposomes are small vesicular systems composed of an amphipathic phospholipid bilayer with an aqueous interior core. They are attractive from a drug delivery perspective due to their varying hydrophobic (membrane) and hydrophilic (core) architecture that allows the

incorporation of both hydrophobic and hydrophilic drugs, including a vast range of antibiotics. Liposomal vesicles vary widely in diameter from 0.025 to 2.5 μm [72] and demonstrate high biocompatibility and biostability resulting in prolonged *in vivo* circulation life [73]. Liposomes are promising molecules for antimicrobial drug delivery as the amphipathic properties of the phospholipids enable strong interactions with the bacterial membranes and enhance the release of the encapsulated drugs across them [74]. Interactions between liposomal vesicles and bacterial membranes occur via multiple mechanisms, including physical adsorption, lipid exchange and fusion. Liposomal-cell interactions are influenced by the composition of the bacterial cell membrane, the exterior structure of liposomal carrier and the biological environment [75].

Fusogenic liposomes are a variation on standard liposomal formulations consisting of inactivated Sendai virus envelope components (mainly for targeting of eukaryotic cells) or nonviral vectors involving the inclusion of specific lipids, for example amphiphilic derivatives of cholesterol including cholesterol hemisuccinate, that increase fluidity of liposomal vesicles to promote weakening of biological membranes. They demonstrate an enhanced ability to fuse with cell membranes, mixing with their lipids architecture, resulting in delivery of vesicular contents into the cytoplasm [72]. They are promising as potential molecules for transversing the Gram-negative outer membrane, enabling delivery of antibiotics such as vancomycin to the periplasmic space. Vancomycin is a glycopeptide antibiotic with a complex chemical structure and a high molecular weight (approximately 1450 daltons). It is used clinically in the treatment of severe, multi-drug resistant Gram-positive infections. It exerts a bactericidal effect by inhibiting the synthesis of peptidoglycan, the major component of the bacterial cell wall [74]. The Gram-negative outer membrane is impermeable to vancomycin macromolecules, therefore they are intrinsically resistant.

Encapsulation of vancomycin within fusogenic liposomes, composed of dioleoylphosphatidylethanolamine, dipalmitoylphosphatidylcholine and cholesterol hemisuccinate, enables delivery to the periplasmic space and therefore allowing activity against Gram-negative bacteria. In a study by Nicolosi and colleagues, non-encapsulated vancomycin demonstrated high MIC values, greater than 512 μg/ml for *E. coli* and *A. baumannii*, which reduced significantly to 6 μg/ml upon inclusion within this liposomal platform [76]. This demonstrates the potential of liposomal drug delivery platforms to extend the therapeutic efficacy of narrow-spectrum Gram-positive therapies.

II. Carbon and peptide nanotubes

Nanotechnologies, for example nanotubes, are at the forefront of research to tackle the most difficult diseases in human and animal health. Nanotubes are materials consisting of hollow cylindrical tubes with nanoscale morphology [77]. Organic-based nanotubes are attracting increased attention for therapeutic applications, with researchers attempting to synthetically replicate the nanoscale architectures of biomolecules such as DNA. Two of the most promising nanomaterial formats are carbon and peptide-based systems [78]. Due mostly to their increased structural strength and biological stability, carbon nanotubes have attracted attention for a range of applications throughout nanomedicine [79]. They can be formed by coiling a single layer of graphene sheet to form single-walled carbon nanotubes, or by rolling several layers to form multi-walled carbon nanotubes. The diameter of single-walled carbon nanotubes varies from 0.4 to 3.0 nm with their length ranging from 20 to 1000 nm. Their formation is driven by van der Waals' intermolecular interactions increasing their structural flexibility. Multi-walled carbon nanotubes are easier to manufacture than single-walled variants, possessing an outer diameter ranging from 2 to 100 nm and inner diameter of 1 to

3 nm respectively. However, their length of 1 to several µm limits their structural flexibility compared to single wall forms. Non-functionalized carbon nanotubes are insoluble in aqueous physiological media making formulation difficult and some concerns do exist regarding their safety in humans. For example, some studies have demonstrated toxicity to mammalian cells, including mediators of the immune response such as macrophages, due mainly to their high hydrophobic character [80].

Carbon nanotubes also lack homogeneity in terms of their size (diameter, length). This makes it difficult to effectively link the type of formulation (e.g. suspension) and concentrations to biological activity [81]. For future antimicrobial drug delivery purposes, carbon nanostructures will likely require functionalization before attachment of a drug and this can prove difficult due to the lack of chemical versatility provided by the rigid carbon-carbon covalent bond. Covalent and noncovalent surface functionalization can be performed on the synthesized carbon nanotubes facilitating the conjugation of antimicrobial agents such as the antifungal amphotericin B [77]. Specific antibacterial activity has also been demonstrated for carbon nanotubes against Gram-negative pathogens including *E. coli*. Single walled nanotubes are particularly effective due to their smaller diameter and therefore increased ability to penetrate the cell wall. Carbon nanotubes display inherent antibacterial activity via physical disruption of *E. coli*'s bacterial cell membrane and oxidation of bacterial glutathione resulting in oxidative stress and cell death [82]. The addition of hydroxyl (-OH) and carboxylic acid (-COOH) groups to the surface of single-walled carbon nanotubes has also been shown to enhance antimicrobial activity against Gram-positive and Gram-negative bacteria. This is due to the formation of cell-nanotube aggregates and subsequent cell wall lysis and DNA release [81]. Interestingly multi-walled carbon nanotubes do not display similar efficacy due to increased length and a reduced ability to aggregate with bacterial cells

[82]. To date the majority of antibacterial carbon nanotube strategies are broad-spectrum including coating with copper to eradicate *E. coli* and *Staphylococcus aureus* [83]. As the Yang group confirmed, neither the difference in cell wall structures between Gram-negative and Gram-positive isolates nor the bacterial cell shape (cocci or rods), alter the effectiveness of the single-walled carbon nanotubes [81]. Carbon nanotube research has therefore been unable to selectively target Gram-negatives but the platform holds great promise in the delivery of current and future drug molecules across the outer membrane barrier.

Peptide-based nanomaterials have also received attention from researchers in the past decade due to their chemical and functional versatility. Peptide nanomaterials possess many advantages compared to current synthetic-based materials utilized throughout healthcare. Peptides possess vast chemical flexibility attributable to variation of the amino acid R-group. As a result they can be utilized to create nanomaterials with very specific functionalities and have the potential to conjugate to a variety of molecules including antimicrobial drugs. Amino acids are the building blocks of peptides, proteins and tissues, existing throughout the body. The primary amino acid sequence of peptides can be modified in order to drive self-assembly to nanomaterials structures (nanofibers, nanotubes) in response to a range of physiochemical stimuli (pH, temperature, ionic strength, presence of specific enzymes). Self-assembling peptide platforms are gaining interest as potential future antimicrobial nanotherapies. The properties required for peptide assembly to occur are similar to those that confer antimicrobial activity to the peptide, namely hydrophobic and electrostatic interactions [84].

Some of the most successful approaches to target Gram-negative bacteria have focused on utilizing self-assembling linear and cyclic peptides. This is due to their ability to target bacterial cell membranes and their structural similarity to naturally occurring polymyxins [85]. Cyclic peptide nanotubes are primarily hexamers or octamers, composed of alternating amphiphilic D- and L-amino acid residues, for example L-tryptophan and D-leucine. They self-assemble into flat ring-shaped structures, with different channel diameters ranging from 0.2 to 1.3 nm [86]. Cyclic peptides can arrange into tubular open-ended structures via intermolecular interactions including hydrogen bonding. When adsorbed onto bacterial cell membranes, they have demonstrated selective membrane permeabilization and lysis of Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) cells compared to mammalian cells [87]. Cyclic peptide nanotubes have great potential as synergistic antimicrobial therapies when used in conjunction with conventional antibiotics. They act as delivery systems increasing antibiotic concentration, hence antimicrobial activity, within the bacterial cell [88].

III. Targeting Gram-negative pathogens with an engineered phage lytic enzyme

Phages are viruses that demonstrate activity against bacterial cells, including multi-drug resistant Gram-negatives. They were originally studied as potential antimicrobial therapies in the United States in the 1930s and more extensively over the past 80 years in Eastern Europe [89]. Phages have been reported to be effective in resolving a variety of infections including: skin infections caused by *Pseudomonas*, *Staphylococcus*, *Proteus*, *E. coli*, surgical wound infections, staphylococcal lung and pleural infections, and *P. aeruginosa* infections in cystic fibrosis patients [90]. There are several reports showing that enzymes isolated from phages, termed lysins, may be considered as therapeutic agents. Lysins produced by bacteriophages are recombinant proteins designed to make “holes” in the cell wall of a bacterium causing rapid cell lysis and death [91]. Until recently the action was mainly restricted to Gram-

649 positive bacteria. Applying the same strategy to Gram-negative pathogens was considered
650 difficult because their enzymatic target, peptidoglycan, is sequestered beneath a protective
651 outer membrane where the lytic enzyme cannot reach. However, research by Lukacik and
652 colleagues demonstrated that phage lytic enzymes can be engineered to cross the outer
653 envelope of Gram-negative bacteria. This is achieved by production of hybrid lysins that have
654 the ability to travel across the outer membrane of Gram-negatives such as *Yersinia pestis* and
655 pathogenic *E. coli* strains, breaking down the peptidoglycan layer in the periplasm. Hybrid
656 lysin demonstrated cidal action against these strains without disrupting the outer membrane.
657 [92]. Variations to this theme also exist. Artilysins are engineered lysins conjugated to
658 cationic peptides extending thire bactericidal activity to Gram-negatives, including *P.*
659 *aeruginosa* and *A. baumannii*. The inclusion of a cationic peptide disturbs the LPS outer
660 membrane layer, allowing lysins to enter the periplasmic space resulting in degradation of
661 peptidoglycan, cell lysis and death [91].

662

663 **Conclusions**

664 The increasing resistance of Gram-negative bacteria to a multitude of currently available
665 antibiotics requires urgent action. Research into novel alternative therapies has focused on a
666 variety of strategies, many of which have failed to progress clinically for patient benefit. The
667 majority of approaches have focused on extending the spectrum of activity of current Gram-
668 positive targeting molecules to Gram-negatives. Whilst this warrants attention and should not
669 be dismissed, a narrow-spectrum, species targeted approach is likely to be more beneficial
670 with greater consideration of a healthy commensal microbiota. This approach requires
671 increased ability to rapidly diagnose and detect specific causative microorganisms implicated
672 infection so that optimal targeted therapy can be provided. The research strategies outlined in

673 this review contribute to expanding potential future therapeutic options at a time when
674 clinical choices are becoming increasingly limited. Currently there are clinical trials
675 involving several antimicrobial peptides. This diverse group of molecules display selective
676 antimicrobial activity against bacteria relative to mammalian cells. Whilst *in vitro* results
677 have demonstrated promise, *in vivo* toxicity and biostability has restricted their progress.
678 Other successful laboratory research, involving attenuation of LPS and inhibition of RND
679 efflux, display promise in limiting the severe clinical implications of Gram-negative
680 infection. Indeed many compounds that display a LPS neutralizing ability may be suitable for
681 future clinical trials as they have demonstrated efficacy both *in vitro* and in animal models.
682 RND efflux pumps inhibitors are attractive compounds that improve the clinical efficacy of
683 antibiotics in resistant bacterial pathogens. Understanding biochemical pathways within
684 Gram-negative bacterial metabolism and resistance will complement the development of
685 novel and tailored therapies. For example, targeted inhibition of DsbA-DsbB enzymes
686 prevents disulfide bond formation and the formation of stable protein tertiary structures
687 within bacterial virulence factors. Despite the promise shown by such an approach no
688 compounds have yet transferred from the laboratory into clinical trials highlighting the
689 importance of pharmaceutical formulation in advancing molecular targets. Improving
690 antibiotic delivery using liposomes or nanotubes is another encouraging approach to extend
691 therapeutic activity of conventional antibiotics to Gram-negatives. There is real hope for
692 progress within this area especially as liposomal approaches have successfully resulted in
693 licensed formulations for a variety of drugs including the antifungal amphotericin B.
694 Engineered lysins have proven to be a truly alternative approach, resulting in a new class of

antimicrobials. However, this still requires further investigation particularly with regard to patient safety and their potential for resistance development.

Future Perspectives

As outlined, the need to eradicate multi-drug resistant bacteria and reduce the growing threat of antimicrobial resistance is an increasing challenge not only for the scientific community but society as a whole. It is everyone's responsibility to use existing antibiotics wisely in order to delay an antimicrobial crisis and allow time for the development of effective novel compounds. The research community has a key role to play in breaking down the microbial processes that lead to resistance and developing strategies to combat such biomolecular pathways. Collaboration is key for successful clinical translation. There is widespread acceptance that a targeted isolate-specific approach to eradicate multi-drug resistant bacteria is necessary to prevent treatment failure and risk of an increased number of antimicrobial resistant strains. Some of the strategies outlined in this review provide great potential for future therapeutics against Gram-negative pathogens. Key to future drug development in this area is repeating the success of the early to mid-20th century boom in antibiotic discovery. Bacteria are the most successful organisms on earth. Just as mother nature provides infectious microorganisms with the tools for survival, so too does she hold the key to solving the riddle of antimicrobial resistance. Scientists at Northeastern University Boston recently uncovered a new antibiotic molecule, teixobactin, produced by bacteria (*Eleftheria terrae*) present in soil. This molecule displays activity against *Methicillin resistant S. aureus* and bacteria implicated in tuberculosis infections but lacks effective action against Gram-negatives. Similarly, "The Drugs from Dirt" project is a worldwide initiative aiming to harness the capability of soil bacteria and the antimicrobial compounds they produce. Microorganisms have long been

known to be capable of producing such molecules. They serve as weapons for survival facilitating destruction of competitive microbial species and enabling survival in natural environments. Therefore their ability to produce Gram-negative selective compounds seems logical. Chemically the most promising of these naturally occurring compounds are peptides. Present throughout the animal and plant kingdoms as part of the immune response, peptides are one the most effective molecules in the fight against multi-drug resistant infection. Most promising, and in contrast with many current therapies, is their ability to attack infectious microorganisms by multiple mechanisms. The ability of bacteria to develop resistance against peptides is thus significantly limited. A mining-like approach is an encouraging strategy to unlock innovative peptide antimicrobials and may eventually lead to an era of discovery: a 21st century "antimicrobial rush." Creating patient friendly therapies, for example oral dose formulations, from the most promising of these molecules will require input from experts within the pharmaceutical industry, healthcare workers and patients themselves. Only this way will such discoveries create true value and easily translate from the laboratory to hospitals, communities and patients.

Executive summary

Introduction

- Resistance to standard therapies employed in Gram-negative bacterial infection has increased to worrying levels over the last 30 years.
- There are a multitude of reasons for the declining clinical translation of antimicrobial drugs in the past 20 years, including safety issues highlighted in clinical trials and concerns from the pharmaceutical industry that investment in novel therapies would not warrant a significant financial return.

The Gram-negative outer membrane as a barrier to therapy

I. Bacterial cell wall structure

- The outer membrane of Gram-negative bacteria acts as a selective barrier to the entry of a vast range of currently available antibiotic molecules.

II. Antimicrobial resistance mechanisms of Gram-negative bacterial cell wall

- Alteration of lipid A, phospholipids and/or protein composition of the outer membrane contributes to increased resistance to antimicrobial/antiseptic molecules that target the bacterial cell membrane.

Strategies for extending therapeutic activity against Gram-negatives

I. Antimicrobial peptides

- Antimicrobial peptides exist throughout nature as mediators of the innate immune response.
- Most cationic antimicrobial peptides target the bacterial cell membrane, leading to rapid cell lysis and bacterial death. They also possess multiple intracellular targets.
- Cyclic antimicrobial peptides, which are among the most promising antimicrobial agents, provide a starting point for designing low molecular mass anti-LPS compounds.

II. Combinational antibiotic treatment for Gram-negative bacteria

- Combination therapy is recommended for patients at high risk of being infected with multidrug-resistant Gram-negative bacteria, demonstrating lower mortality rates and improved clinical outcomes.

III. The activity of silver against Gram-negative bacterial infection

- Silver increases the permeability of Gram-negative bacterial membranes and can potentiate the activity of a broad range of antibiotics against these microorganisms.
- Silver nanoparticles have attracted interest due to their potential applications as wound dressings, medical device coatings, and within drug delivery.

Specific methods to target Gram-negative pathogens

I. Negating the biological effects of Gram-negative lipopolysaccharide

- An important consideration when treating suspected or confirmed Gram-negative infection is preventing the biological effects of Gram-negative lipopolysaccharide. This potent molecule signals bacterial invasion and triggers defensive host responses to release pro-inflammatory mediators, cytokines, chemokines and lipoproteins.

II. Targeting disulfide bond formation by the bacterial DsbA-DsbB enzyme system of Gram-negative bacteria

- DsbA-DsbB system in Gram-negative bacteria is a key target for the development of new drug molecules. Inhibition of disulfide bond formation has been demonstrated to prevent the assembly of key bacterial virulence factors.

III. Inactivating Gram-negative efflux pumps

- Inactivating Gram-negative efflux pumps has the potential to restore resistant antibiotics activity.

Methods to extend the spectrum of activity of existing narrow-spectrum Gram-positive antibiotics to Gram-negatives

I. Fusogenic liposomes

- Encapsulating narrow-spectrum Gram-positive selective antibiotics within fusogenic liposomes has been shown to broaden their spectrum of activity to cover Gram-negative infections by enabling transversion across the outer membrane.

II. Carbon and peptide nanotubes

- Single-walled carbon nanotubes may be useful as future antimicrobials due to their inherent antimicrobial properties and ability to deliver existing and future antibiotic molecules via nanoparticle-based drug delivery.
- Cyclic D, L-alpha peptides are able to selectively target bacterial cell membranes, including the outer membrane of Gram-negatives. They are able to self-assemble, forming peptide nanotubes with the potential to act as biofunctional nanomaterials and improve intracellular delivery of antibiotics.

III. Targeting Gram-negative pathogens with an engineered phage lytic enzyme

- Phage lytic enzymes can be engineered to cross the outer envelope of targeted Gram-negative bacteria. This is achieved by production of a “hybrid lysin” and “artilysin” that have the ability to kill pathogenic strains of *E. coli* and *P. aeruginosa*.

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